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The effects of carbohydrate and HMB supplementation on glycogen synthesis post-exercise

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The effects of carbohydrate and HMB supplementation on glycogen synthesis post-exercise

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Thesis

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Master of Science in Kinesiology

The University of Texas at Austin

August 2013

Acknowledgements

Above all, I am grateful to the almighty God for establishing me to complete this thesis. I wish to express my sincere thanks to my advisor, Dr. John Ivy, for his excellence guidance, caring and support. Without his guidance and persistent help, this thesis would not have been possible. I would like to thank Zhenping Ding for teaching me a lot of techniques and for providing me with his assist during my Master's degree. I would also like to thank all my colleagues in Exercise Physiology and Metabolism Laboratory. I received generous support from them and I would never have been able to finish my thesis without them.

Lastly, I would like to deeply thank my family for their support, encouragement, and prayer.

Abstract

The effects of carbohydrate and HMB supplementation on glycogen synthesis post-exercise

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Carbohydrate plus additional protein supplementation provided immediately after exercise has been found to increase the rate of muscle glycogen restoration compared to carbohydrate alone. To examine whether leucine, and/or β-hydroxy-β-methylbutyrate (HMB) to carbohydrate plus protein supplementation affects short-term recovery (45 min) of muscle glycogen, we compared plasma glucose and insulin, the muscle glycogen concentration, and the cellular signaling proteins controlling muscle glycogen synthesis 45 min after supplementation.

Rats (n=35) underwent high-intensity resistance exercise followed by supplementation with carbohydrate (CHO: 1.2g/kg body weight), carbohydrate with whey protein (CP: 1.2g CHO + 375mg whey protein/kg body weight), carbohydrate with whey protein plus HMB (CPH: 1.2g CHO + 375mg whey protein + 400mg HMB/kg body weight), carbohydrate with whey protein, HMB plus leucine (CPHL: 1.2g CHO + 375mg whey protein + 400mg HMB + 444mg leucine/kg body weight) or exercise only (CON). Blood samples were collected immediately after exercise and 45 min after

supplementations. Muscle samples of plantaris were excised immediately and 45 min post-exercise. Plasma glucose was increased by CHO and CPH supplementation and reduced by CPHL at 45 min post-exercise. Plasma insulin was elevated by CP and CPHL treatments compare to CHO. Muscle glycogen concentration was unaffected by all treatments and did not differ from CON. Phosphorylation of Akt/PKB, GSK3α/β, and GS at 45 min of recovery for all supplements was not significant difference from CON. Phosphorylation of mTOR was significantly increased by CPHL and CP supplementation compared to CON, CHO, and CPH. Phosphorylation of AS160 was markedly reduced by CPH supplementation compared to CON. These results suggest that supplementing with carbohydrate plus protein with or without leucine and its metabolite, HMB, to enhance muscle glycogen replenishment following exercise may not provide an advantage during the early phase of recovery (45 min). Furthermore, there is some indication that HMB may elicit insulin resistance, and this needs further evaluation.

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Introduction

Muscle glycogen is a significant determinate of athletic performances. Utilization of intramuscular glycogen is tightly related to exercise intensity. For example, muscle glycogen will be quickly depleted when individuals perform exercise at higher intensity as opposed to lower exercise intensity. Consequently, the depletion of muscle glycogen leads to fatigue and impairs the athletes' ability to perform. Therefore, fast recovery of intramuscular glycogen stores through nutritional supplements has received great attention as a strategy for athletes and physically active individuals in order to maintain and improve their physical performance and exercise training.

The availability of glucose and insulin determine in large part the rate of muscle glycogen synthesis. Among the diverse nutrients that we consume through our daily meal, carbohydrate (CHO) is considered the primary nutrient to enhance the rate of muscle glycogen synthesis. CHO mainly supplies glucose, which compose the monomers that makes up glycogen, and stimulates the secretion of insulin. Ivy (1998) found that ingestion of 1.2 to 1.5 g of CHO /kg body weight could maximize the rate of muscle glycogen restoration when the supplement was provided immediately after exercise and then at 2-hour intervals for up to 6 hours post-exercise.

Later research pointed out the importance of additional protein to a carbohydrate supplement for acceleration of muscle glycogen recovery. Although the mechanisms of additive protein have not clearly been identified, several studies showed that additional protein with carbohydrate supplement enhanced insulin secretion (Zawadzki et al. 1992, Berardi et al. 2006). Although Zawadzki et al. (1992) found that the addition of protein increased insulin secretion more than carbohydrate alone. Ivy et al. (2002) did not find a difference between carbohydrate plus protein and carbohydrate alone and insulin

response. However, carbohydrate and protein supplementation did result on a greater rate of glycogen synthesis. Therefore, it was suggested that additional protein might play a critical role in activation of enzymes regulating glycogen synthesis beyond the increase of insulin alone.

In accordance with these findings, we hypothesized that different combinations of protein and their metabolites with carbohydrate would be more effective in activating key enzymes of cell signaling pathways controlling muscle glycogen synthesis than carbohydrate alone. We used whey protein, leucine, and β-hydroxyl-β-metylbutyrate (HMB), which is a metabolite of leucine. Whey protein is recognized as a high quality protein containing a rich source of branched-chain amino acids (BCAA). Moreover, carbohydrate supplementation with whey protein has been shown to increase the rate of muscle glycogen recovery in skeletal muscle (Morifuji et al. 2010). Leucine has also been demonstrated to positively potent promote glucose uptake under insulin-free conditions (Iwanaka et al. 2010, Doi et al. 2005, Nichitani et al. 2002). These studies suggested that leucine increased glucose uptake through an insulin-independent pathway, possibly the mTOR signaling pathway.

Although the effects of HMB supplementation on glycogen synthesis in skeletal muscle are unknown, HMB has been reported to prevent muscle damage and to preserve anabolic conditions after exercise. However, recent research suggests that it may cause insulin resistance (Gerlinger-Romero et al. 2011). Therefore, it becomes important to determine the effect of HMB in combination with carbohydrate and protein on glycogen recovery post-exercise.

Methodology

ANIMAL CARE AND HOUSING

Young male Sprague-Dawley rats (n=35), 2-3 months old, were obtained from Charles River Laboratories (Wilmington, MA). They were randomly housed 2 per cage in an animal facility kept at a constant temperature of 21°C and on a 12 h dark-light cycle schedule from 7:00 AM to 7:00 PM. They were fed standard laboratory chow (Prolab RMH 1800 5LL2, LabDiet, Brentwood, MO) and water ad libitum. Rats were allowed to acclimate to their environment once they arrived on campus. The Institutional Animal Care and Use Committee at The University of Texas at Austin approved all of the animal experiments and procedures.

EXPERIMENTAL DESIGN

There were 5 treatments groups (n=7) including a control group (CON) that was immediately sacrificed after exercise. After one week of acclimation, the rats were randomly separated into the 5 different groups according to their bodyweight, and then started a familiarization procedure. All treatment groups received different supplements after exercise except CON group. The treatment solutions consisted of 1) control (CON: sacrifice immediately after exercise with no treatment solution), 2) carbohydrate (CHO: 1.2g/kg body weight), 3) carbohydrate with whey protein (CP: 1.2g CHO + 375mg whey protein/kg body weight), 4) carbohydrate with whey protein plus HMB (CPH: 1.2g CHO + 375mg whey protein + 400mg HMB/kg body weight), 5) carbohydrate with whey protein, HMB plus leucine (CPHL: 1.2g CHO + 375mg whey protein + 400mg HMB + 444mg leucine/kg body weight). Krebs-Henseleit Buffer (KHB) adjusted to a pH 7.4 was used to make all solutions. For supplying supplements, 4.5g of CHO (α-D-Glucose,

anhydrous 96%, ALDRICH, #14417TH), 1.41g of whey protein (SI03, Inc., Cape Girardeau, MO, U.S.A.), 1.88g of Ca(HMB)2 • H2O (Abbott Nutrition, Columbus, Ohio, U. S. A.), 1.67g of leucine (Ajinomoto, Fort Lee, NJ, U.S.A.) was added to 30ml of KHB. 8ml/kg body weight of treatment incubated through gavage.

ANIMAL FAMILIARIZATION TO RESISTANCE EXERCISE

For 2 weeks, all five groups of rats were familiarized with the ladder climbing protocol. Rats were trained three non-consecutive days a week. For the first week of training, the rats underwent three familiarization training sessions. Each session consisted of 4 climbs without weight attachment. The second week of training, proper weight (from 50% to 70% of rat's body weight) was attached to their tails. This weight was continually increased by 10% of body weight per each session. In addition, the number of repetitions (climbs) also incremented 8 times per session. Two-minute breaks were given between every climb. After the last familiarization session, the rats were given 2 days of rest before the experiment was started.

EXPERIMENTAL PROTOCOL

On the experimental day, the rats fasted for 12-14 hours and then climbed the ladder with the attachment, which consisted of 75% of their body weight attached to their tail. They went up the ladder 10 times and had a 2 minute break between climbs. Blood samples (0.5ml) were collected into a 1.5 ml test tube containing 50µl of EDTA (24mg/ml, pH 7.4) before the rats received treatment and after 45 minutes of incubation. The collected blood samples were placed on ice until centrifuged. After the first blood collection, all groups, except CON, received their respective supplement via gavage.

After 45 minutes, the second blood sample was taken. All blood samples were centrifuged at 4,000g for 15 minutes at 4°C and then stored at -80°C for plasma glucose and insulin analysis. Immediately after taking the second blood sample, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (75mg/kg of body weight). Muscle samples of plantaris were collected. The muscle samples were frozen in liquid nitrogen, and stored at -80°C for later analysis. Right after all samples were collected, the rats were euthanized through a cardiac injection of sodium pentobarbital (65mg/kg of body weight).

PLASMA GLUCOSE ANALYSIS

Plasma glucose was determined by using a glucose color reagent kit (Raichem, San Diego, CA). following the manufacture's instruction, 10µl plasma samples were put into tubes containing 1.5ml of reagent. After 10 min of incubation at 37°C, all samples were visualized at wavelength of 500 nm by a Beckman DU 640 spectrophotometer.

PLASMA INSULIN ANALYSIS

A commercially available radioimmunoassay (RIA) kit (Linco Research St, Charles, MO) was used to measure plasma insulin following the manufacture's instructions. ^{125}I-labeled rat insulin was delivered to the tubes containing standards, controls, or plasma samples. The tubes were incubated overnight at 4°C. All samples measured on a Wallac 1470 WizardTM automatic gamma counter (Pekin Elmer life sciences, Truku, Finland). The company provided the standards for calculation of insulin concentration.

MUSCLE GLYCOGEN ANALYSIS

Muscle glycogen was determined by the method described by Passonneau and Lauderdale (1974). Around 80mg of muscle was digested in 1N KOH at 65-70°C for 30 min. After muscle digestion, 100µl of the homogenates were added to tubes containing 250µl of 0.3 mol/L sodium acetate buffer (pH4.8). Next, additional 10µl of 50% glacial acetic acid and 250µl of 0.3 mol/L sodium acetate buffer containing 10mg/ml amyloglucosidase were delivered to the tubes. All tubes were incubated overnight at room temperature. After overnight incubation, 25µl of 1N NaOH were put to the sample tubes for neutralization. Glucose concentration was measured by using a glucose color reagent (Raichem, San Diego, CA).

MUSCLE HOMOGENIZATION

Frozen muscle samples (80-100µg) were homogenized in ice-cold homogenization buffer (consisting of 20mM EGTA 50mM NaF, 100mM KCl, 0.2mM EDTA, 50mM glycerophosphate, 1mM DTT, 0.1mM PMSF, 1mM Benzamidine, 0.5mM Na Vanadate) with a glass tissue pestle (Corning Life Science, Acton MA). The muscle homogenates were centrifuged at 13,000g for 15minutes at 4°C. The centrifuged samples were stored at -80°C for later analyses after aliquoting the homogenate into several microfuge tubes. Protein quantification of muscle samples was measured through a modification of the Lowry protein assay method (Lowry et al. 1951).

WESTERN BLOTTING

Western blots were run to determine the phosphorylation state of Akt/PKB, mTOR, AS160, GS, and GSK3α/β. α-tubulin was measured as an internal control.

Homogenized muscle samples (100µl) were diluted with an equal amount (1:1) of Laemmli buffer (1.25M Tris, 20% glycerol, 4% SDS, 0.02% bromophenol blue, 10% β-meraptoethanol, pH 6.8) and denatured at 95°C for 10 minutes. For all blots wells were loaded with 60µg protein. The western blots were run on 10% gels of sodium dodecyl sulfate polyacrylamide for 90 minutes at 130V. After completion of electrophoresis, resolved muscle proteins were transferred to a nitrocellulose membrane at 90V for 90 minutes. The transfer membranes were blocked in 7% non-fat dry milk dissolved in Tris-Tween 20 buffered saline (TTBS) for 30 minutes at room temperature (RT). Next, the blocked membranes were washed 3 times with TTBS for 5 minutes and then incubated overnight with either phosphor-Akt/PKBSer473, anti-phospho-mTORSer2448, anti-phospho AS160Thr642, anti-phospho-GSSer641, anti-phospho-GSK3α/βSer21/9, or anti- α-tubulin (Cell Signaling Technology, Inc., Danvers, MA) at 4°C. All primary antibodies were prepared 1:1000 with 2% non-fat dry milk dissolved in TTBS.

After an overnight incubation, species-specific (anti-rabbit) immunoglobulin G (IgG) secondary antibodies (Cell Signaling Technology, Inc., Danvers, MA) were applied to the membranes for 1 hour at RT following 3 times washing for 5 minutes each time. The secondary antibodies were diluted to 1:1000 in 2% non-fat milk TTBS. After membrane washing, the proteins were detected by a ChemoDoc ECL detection system and quantified by Quantity One software in accordance to the manufacturer's instructions (Bio-Rad, Hercules, CA).

STATISTICAL ANALYSIS

All muscle data were analyzed by a one-way ANOVA. A two-way repeated ANOVA was performed on plasma glucose and insulin data. All statistical results were

confirmed using PASW v20.0 software (PASW Inc., Chicago, IL) and significance was set at $p<0.05$. All data are expressed as means ± standard error (SE).

Results

ANIMAL CHARACTERISTICS

A total of 35 rats were involved in the present study. Body weight of each group was CON 402.43 ± 11.74g; CHO 401.86 ± 7.01g; CP 409.29 ± 7.58g; CPH 392.86 ± 8.22g; CPHL 403.71 ± 5.85g. There were no significant differences among groups in regard of body weight.

PLASMA GLUCOSE LEVEL

The results for plasma glucose level are shown in Fig. 1. There were no differences in plasma glucose, immediately after exercise among all groups. There were significant differences among groups at 45 min after receiving treatment. CHO and CPH treatments elevated plasma glucose 45ming after supplementation. In contrast, plasma glucose significantly decreased at 45 min after supplementation with CPHL (Fig. 1A). Moreover, the glucose area under the curve (AUC) was significantly decreased with the CPHL treatment, but increased with CHO, CP, and CPH treatment (Fig. 1B).

PLASMA INSULIN LEVEL

The level of plasma insulin is illustrated in Fig 2. Plasma insulin level immediately after exercise showed no difference among treatment groups. After 45min of supplementation, all treatments raised the plasma insulin level (Fig. 2A). CPHL significantly increased the plasma insulin level compared to CHO. CP also elevated the plasma insulin level relative to CHO at 45 min post supplementation. There was no significant difference in insulin AUC among all treatment groups (Fig. 2B)

MUSCLE GLYCOGEN CONCENTRATION

Fig. 3 shows the concentration of muscle glycogen post-exercise after 45 min of supplementation. There was no significant difference among treatment groups observed. Nor did any of the treatment groups differ from CON.

MUSCLE PROTEIN SIGNALING RESPONSES

Phosphorylation of Akt/PKB (Ser473)

Phosphorylation of Akt/PKBSer473 (Fig. 4) following supplementation was not different from CON and there was no treatment effect.

Phosphorylation of mTOR (Ser2448)

mTOR phosphorylation was markedly increased 45 min post CPHL supplementation compared to CON, CHO and CPH supplementation in plantaris muscle (Fig. 5). In addition, CP significantly enhanced mTOR phosphorylation compared to CHO.

Phosphorylation of AS160 (Thr642)

AS160 phosphorylation was considerably decreased after 45 min post CPH supplementation compared to CON (immediately after exercise) (Fig. 6). Furthermore, phosphorylation of AS160 showed a tendency to be reduced by CPHL treatment below CON, but this difference was not significant.

Phosphorylation of GSK3 α/β (Ser21/9)

Neither GSK α or β phosphorylation was statistical difference from CON at 45 min following post-exercise treatment in plantaris muscles (Fig. 7). Furthermore, there were no significant differences among treatments.

Phosphorylation of GS (Ser641)

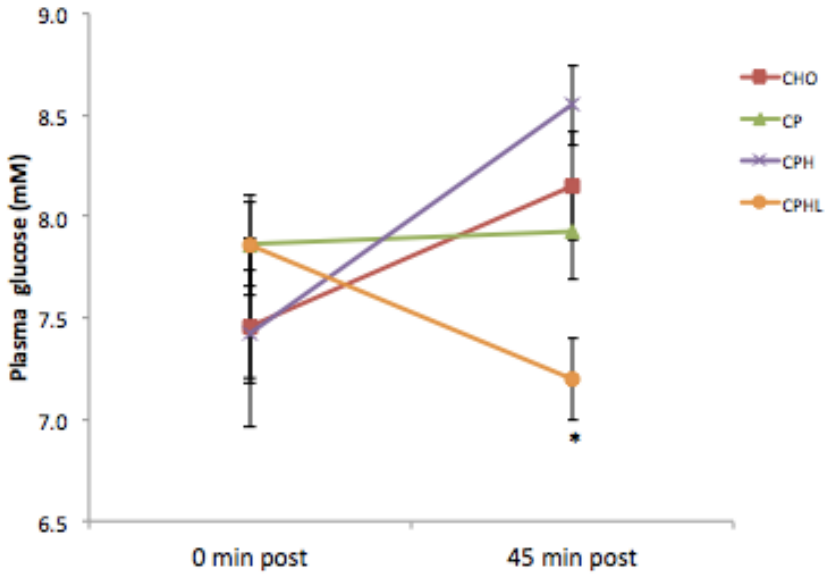
GS phosphorylation was unaffected by the treatments 45 min after supplementations in plantaris muscle (Fig. 8).

Abundance of total α-tubulin

Total α-tubulin was measured as an internal control protein. As shown Fig. 9, α-tubulin did not show any statistical differences across treatments, which suggests equal amounts of total protein loading for all treatments.

FIGURES

A



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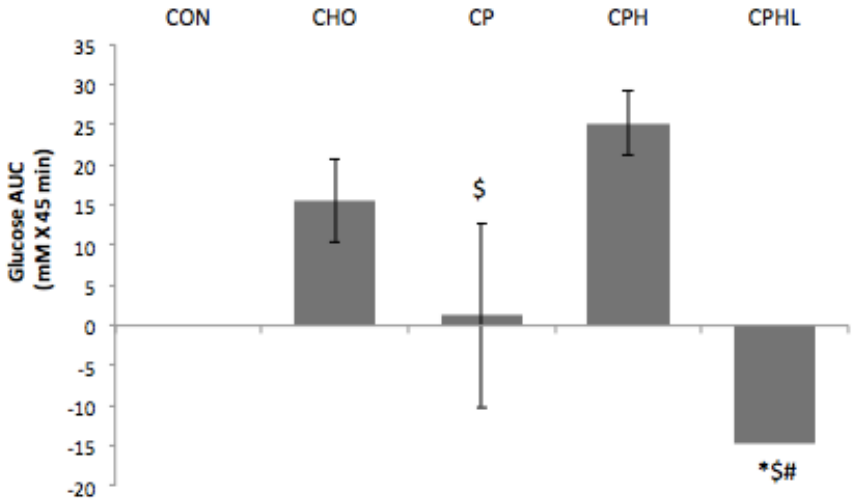
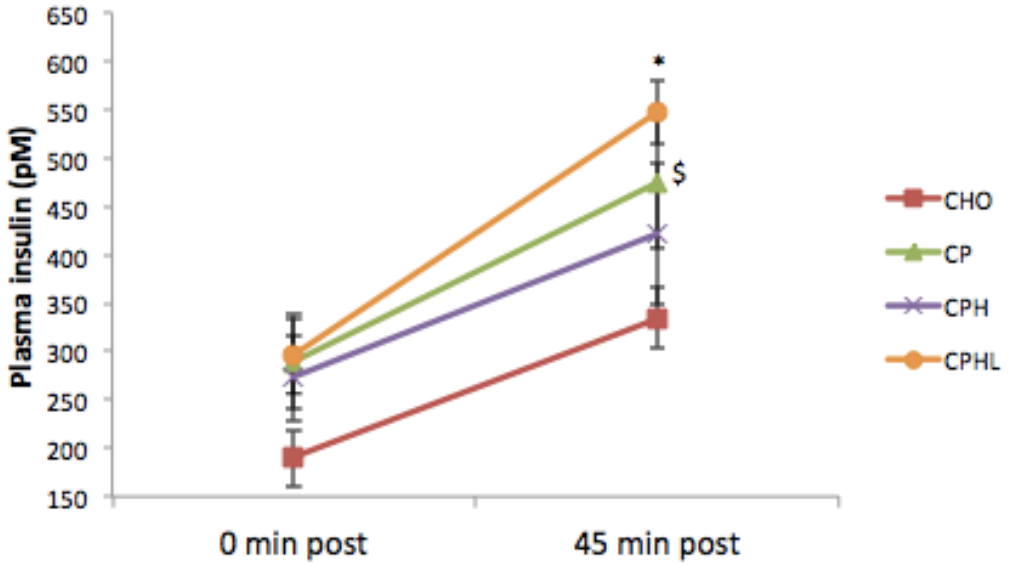


Figure 1. (A) Plasma glucose immediately after exercise, and 45 min post supplementation. Values are means ±SE. *, p<0.05 CPHL vs. all other treatments at the same time point. (B) Plasma glucose AUC. Values are means ±SE. *, p<0.05 vs. CHO. $, p<0.05 vs. CPH. #, p<0.05 vs. CP

A



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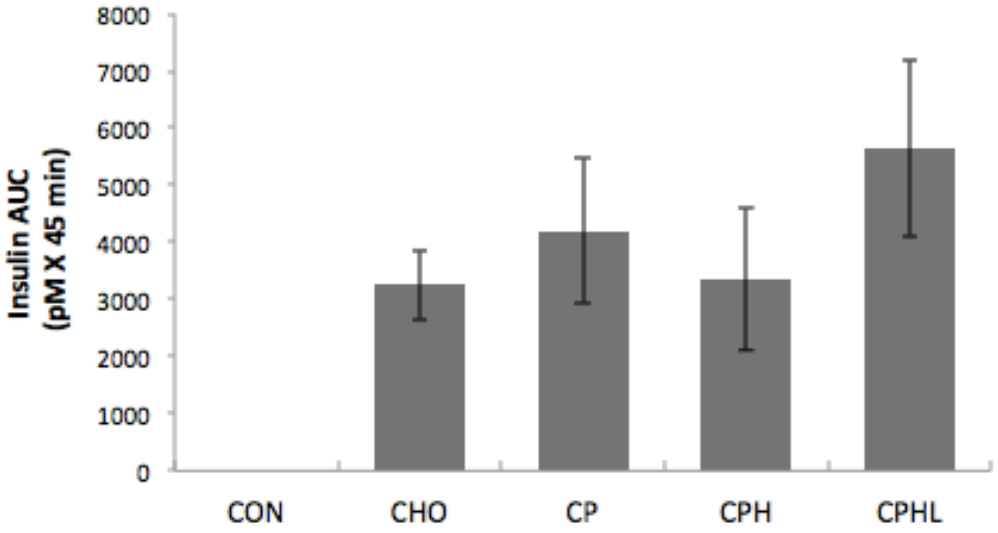


Figure 2. (A) Plasma insulin immediately after exercise, and 45 min post supplementations. *, p<0.05 CHO vs. CPHL at 45 min. $, p<0.05 CHO vs. CP at 45 min. (B) Plasma insulin AUC. Values are means ±SE.

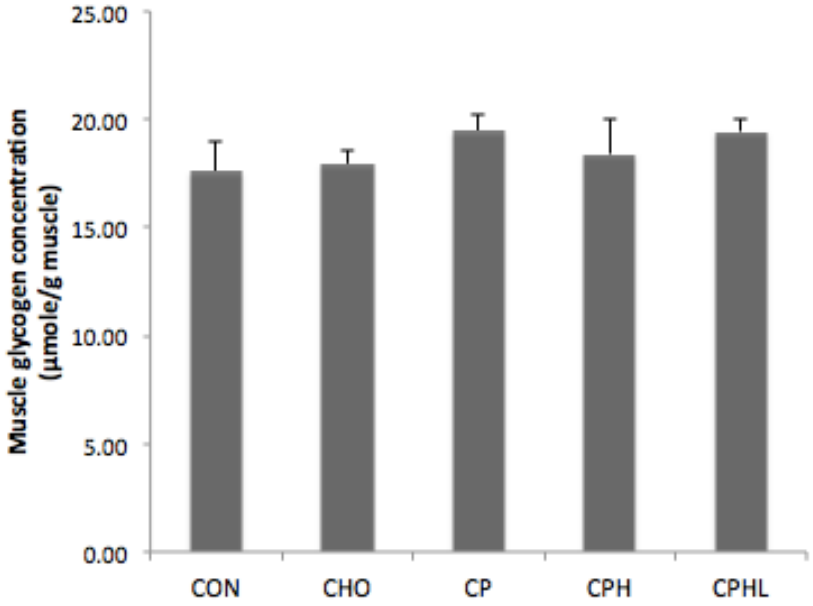


Figure 3. Muscle glycogen concentration 45 min after each treatment. Values are expressed means ±SE. No significant difference was detected.

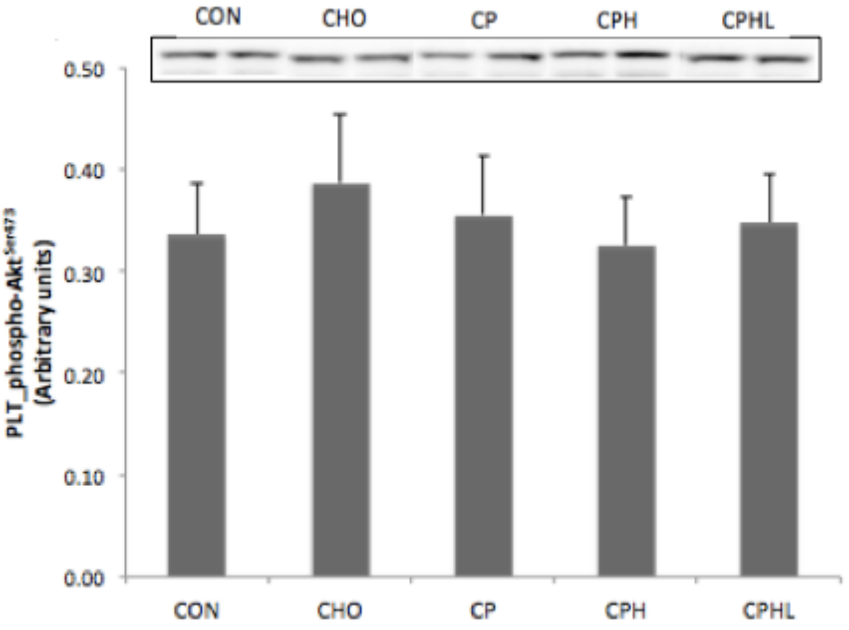


Figure 4. Plantaris Akt^{Ser473} phosphorylation 45 min after each treatment. Values are means ±SE.

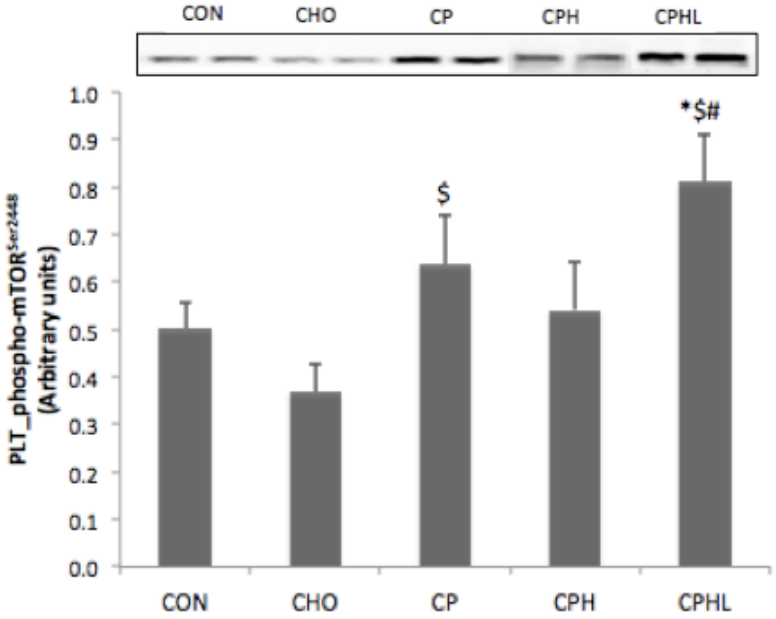


Figure 5. Plantaris mTOR^{Ser2448} phosphorylation 45 min after each treatment. Values are means ±SE. *, $p<0.05$ vs. CON. $, $p<0.05$ vs. CHO. #, $p<0.05$ vs. CPH

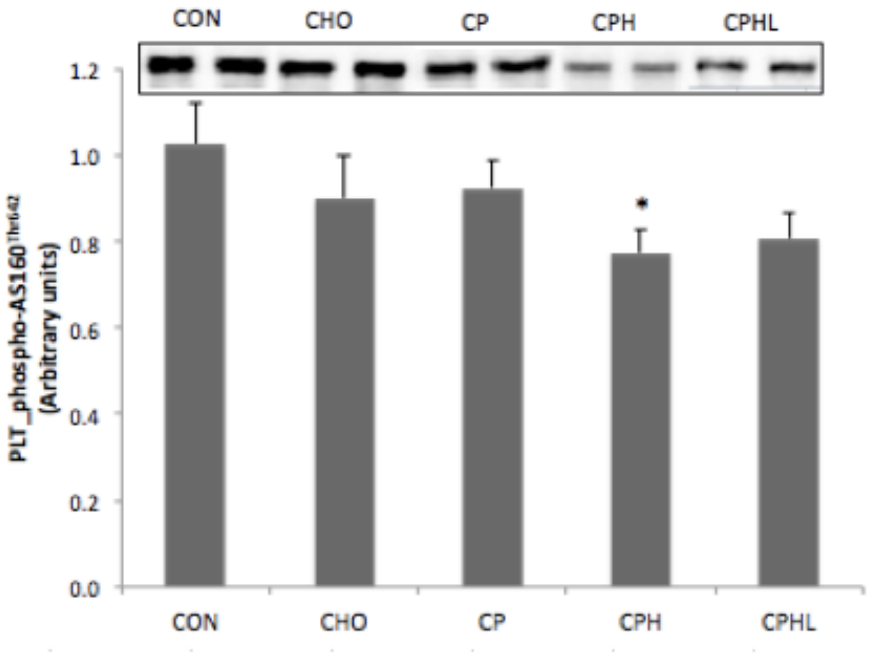
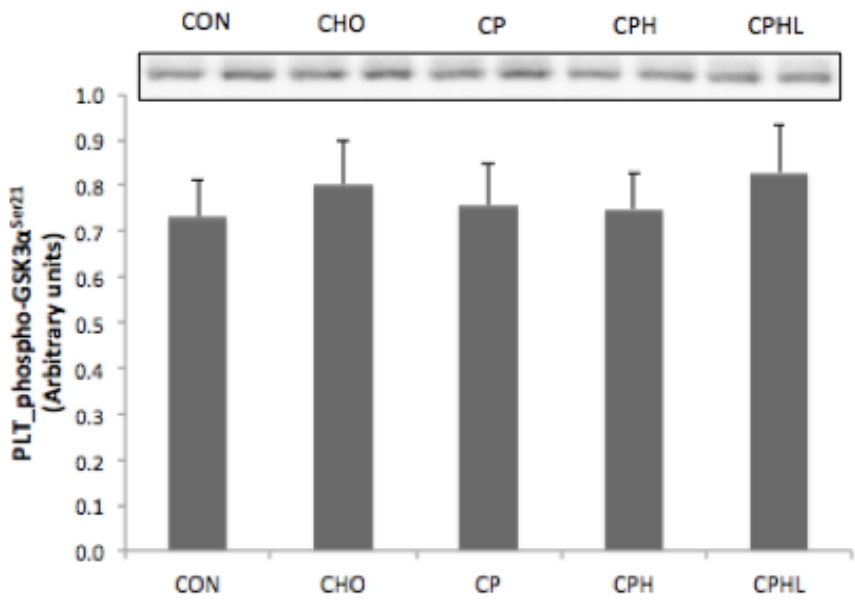


Figure 6. . Plantaris AS160^{Thr642} phosphorylation 45min after each treatment. Values are means ±SE. *, $p<0.05$ vs. CON.

A



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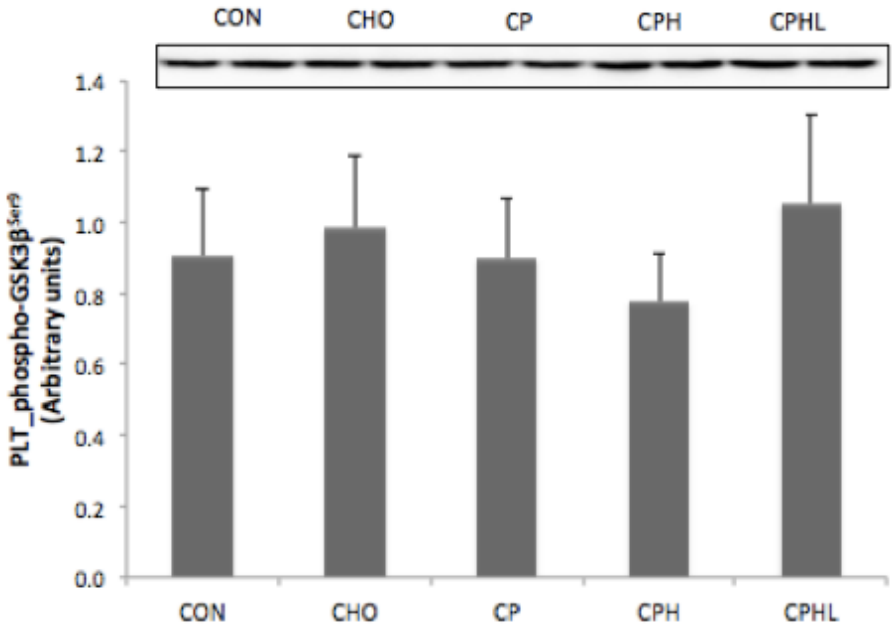

Figure 7. Plantaris GSK3 (A) α^{Ser21} and (B) β^{Ser9} phosphorylation 45 min after each treatment. . Values are means ±SE.

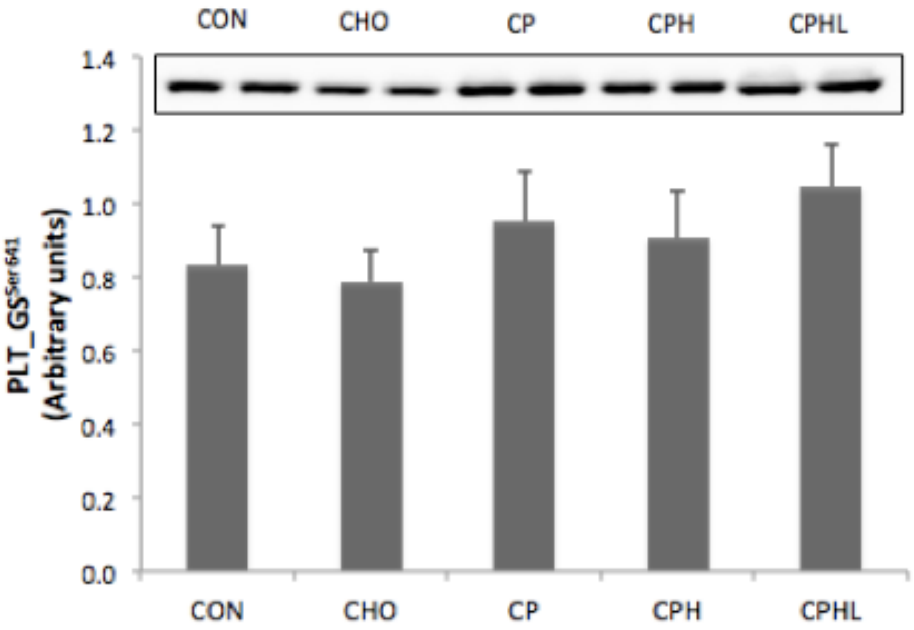

Figure 8. Plantaris GS^{Ser641} phosphorylation 45 min after each treatment. Values are means ±SE.

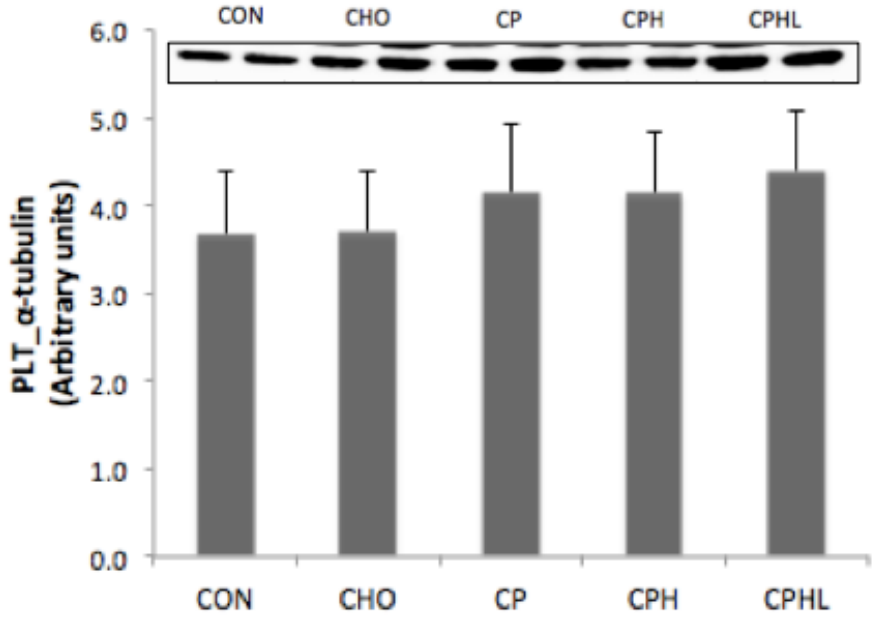

Figure 9. Total protein abundance of α-tubulin in plantaris was used to evaluate the amount of protein equally loaded. Values are expressed as means ±SE.

Discussion

In the present study, carbohydrate alone or carbohydrate with additional protein and its metabolites supplementations provided immediately after exercise changed plasma glucose and insulin levels. Nevertheless, they did not affect muscle glycogen replenishment during 45 min of recovery. In addition, the phosphorylation status of cell signaling proteins controlling muscle glycogen synthesis, such as Akt/PKB, GSK3 α/β, and GS, did not show a significant difference among all treatments compared to CON. However, CPHL supplementation enhanced the phosphorylation of mTOR compared to CON, CHO, and CPH supplementation during the 45 min recovery period. CP treatment also increased the phosphorylation of mTOR more than CHO alone. In addition, CPH supplementation reduced the phosphorylation of AS160 compared to CON during early recovery.

The availability of glucose and insulin post-exercise is considered a primary stimulus for muscle glycogen resynthesis. Both factors are able to accelerate glucose uptake not only by the translocation of GLUT 4 (Kuo et al. 1999) but also by the activation of anabolic signaling pathways (Bak et al. 1991). In the present study, CP and CPHL supplementations greatly promoted the secretion of insulin over CHO alone at 45 min post-exercise. Zawadzki et al. (1992) reported that carbohydrate plus protein treatment improved the rate of muscle glycogen synthesis during 4-h recovery via increasing insulin secretion caused by additional protein. However, several investigators criticized this study. Because the supplements were not isoenergetic, higher calorie count could possibly accounted for the elevated insulin level after carbohydrate plus protein supplemention. In a more recent study by van Loon et al. (2000) similar results to Zawadzki et al. (1992) was found even though they compared isocaloric treatments

between carbohydrate (1.2 g carbohydrate•kg-1•h-1) alone and carbohydrate (0.8 g carbohydrate•kg-1•h-1) plus protein (0.4 g protein•kg-1•h-1). In our study, we observed a remarkable increase in the level of insulin during 45 min post-exercise recovery after ingestion of CP and CPHL compared with CHO alone. However, these results did not result in an increased muscle glycogen concentration in this early recovery period. This discrepancy is probably caused by a high-amount of carbohydrate (1.2 g carbohydrate•kg-1•h-1) that we used. The study from van Hall et al. (2000) demonstrated that carbohydrate plus protein supplement did not amplify leg glucose uptake compared to carbohydrate alone even though the insulin level was increased by carbohydrate plus protein supplement. This study suggested that effects of elevated insulin had no benefit when carbohydrate was provided at rates of 1.0 g carbohydrate•kg-1•h-1. Additionally, Jentjens et al. (2001) reported that additional protein to a high-amount of carbohydrate (1.2 g carbohydrate•kg-1•h-1) supplementation did not result in a further increase in the rate of muscle glycogen replenishment. Taken together the results of our present study and the other studies (van Hall et al. 2000, Jentjens et al. 2001) imply that the availability of protein and its effect on muscle glycogen synthesis post exercise may be related to the amount of glucose made available.

The present study showed that CHO and CPH treatments increased the level of plasma glucose compared to CP and CPHL during the 45 min post-exercise period. Although we assumed that abundant glucose availability after exercise is crucial for promoting glycogen resynthesis, our results did not affirm this idea. The recovery period around 30-60min post-exercise is known as an insulin-independent glycogen synthetic phase. This phase is mainly controlled by exercise-induced GLUT 4 translocation (Thorell et al. 1999) and insulin is not required. Similar to the results of Hara et al. (2011), the present study did not find any differences in the muscle glycogen

concentration at 45 min after all treatment groups. However, Hara et al. (2011) observed a significant improvement of muscle glycogen at 90min after ingestion. These results suggest that nutritional supplementation could be beneficial in an insulin-dependent phase, which begins at least after 1-h post-exercise, but not in the insulin-independent phase.

With regard to the activation of cell signaling proteins regulating muscle glycogen synthesis, CPHL and CP treatments strongly improved the phosphorylation of mTOR relative to CON, CHO, and CPH, respectively. The mTOR pathway can independently activate glucose transport. Phosphorylated mTOR subsequently activates p70S6K, which is one of its down stream substrates. p70S6K is then able to inactivate GSK3 by phosphorylation, which leads to the activation of GS by dephosphorylation (Sutherland et al. 1993, Armstrong et al. 2001). Although CPHL and CP supplementation induced mTOR phosphorylation, there were no significant difference detected in the phosphorylation of GSK3 α/β and GS. Hara et al. (2011) also showed no effects of GSK α/β at 30 min post-exercise supplementation compared to control group. However, they observed a significant difference in the dephosphorylation of GS after 30 min post-exercise supplementation compared to a control group. This difference in results may be related to the different exercise modes, exercise intensities, and durations of exercise used to deplete the muscle glycogen stores. Hara et al. (2011) used a strenuous 3-h swimming program, whereas in the current study a resistance exercise challenge was used.

Leucine is a popular branched-chain amino acid because it can directly stimulate mTOR phosphorylation. In addition, leucine could have benefits to improve glucose uptake after exercise via the activation of mTOR/p70S6K (Iwanaka et al. 2010) or PI3K and PKC pathways (Nichitani et al. 2002). In the present study, we cannot determine if

leucine improved glucose uptake because we provided leucine in combination supplement ingredients and had no direct measure of rate of glucose uptake. Although there was no marker of glucose uptake, we assessed the phosphorylation of AS160, which provides an indirect measure of the activation of GLUT 4. After 45 min post CPHL supplementation, AS160 phosphorylation was reduced below CON, but this reduction was not significant. Future work is needed to clarify if leucine is involved in suppressing AS160 phosphorylation by CPHL supplementation or if other ingredients in the supplement are involved such as HMB.

HMB is a metabolite of leucine, and it has been reported that HMB actively inhibits protein degradation in skeletal muscle when the body goes through catabolic stresses, including cancer and AIDS (Wilson et al. 2008). Furthermore, HMB is considered a recovery agent following high intensity or prolonged exercise (Nissen et al. 1996, Wilson et al. 2009). The relationship between HMB and muscle glycogen replenishment has received less attention. In the present study, acute HMB combined with carbohydrate, whey protein, with or without leucine treatment did not affect muscle glycogen concentration during 45 min of post-exercise recovery. However, CPH group significantly reduced the phosphorylation of AS160 compared to CON at 45 min post supplementation. This result might relate to the findings of Vukovich et al. (2001) and Gerlinger-Romero et al. (2011).

Vukonich et al. (2001) determined that HMB kinetics peaked within 60-120 min whether 1g or 3g of HMB was consumed with or without glucose. Moreover, this study demonstrated that ingestion of HMB plus glucose could delay peak HMB metabolism relative to HMB alone. This study might suggest that we need to set a longer time point of at least over 60 min in order to observe any effects of HMB.

Another possibility comes from the study of Gerlinger-Romero et al. (2011) indicating that chronic HMB supplementation for 4 weeks study evoked insulin resistance. Although in the present study CPH supplementation tended to increase plasma glucose relative to CHO supplementation even both group showed the plasma insulin response, the difference did not reach significance. Although we cannot conclude that acute HMB causes insulin resistance, it would be of interest to examine how HMB combined with carbohydrate supplementation affects glucose and insulin metabolism and its effects on glycogen synthesis.

In conclusion, our results demonstrated that combinations of carbohydrate and additional protein and its metabolites did not affect muscle glycogen replenishment at a very early recovery period (45 min post-exercise) although some of our treatments did increase insulin secretion above CHO alone. These results suggest that post-exercise nutritional supplementation may not significantly affect muscle glycogen restoration during the insulin-independent phase (after 30-60 min post-exercise) of glucose uptake. Therefore, future research should evaluate the effect of these treatments over a longer recovery time period of at least after 90 min. Furthermore, it would be important to assess the various combination of whey protein, leucine, and/or HMB with or without carbohydrate supplements in order to determine the effect of each metabolite on cellular signaling pathways regulating muscle glycogen synthesis.

Background literature

INTRODUCTION

Muscle glycogen is an essential fuel of athletic performances. This fuel source is mainly utilized at moderate- to high-intensity exercise. When the amount of muscle glycogen is lowered, athletes or people who enjoy exercise will not continue their performance because of fatigue. In addition, high muscle glycogen storage has been shown to improve training quality. Therefore, researchers have focused on muscle glycogen restoration by nutritional supplements as an effective strategy in order to promote performance among athletic or physically active populations.

In the past, endurance types of exercise were conducted to study muscle glycogen depletion. These types of exercise are easily controlled to reach 70-80% of $VO_{2max,}$ which is an intensity that progressively uses muscle glycogen for energy generation. However, recent studies have identified that intensive resistance exercise actively utilizes muscle glycogen when exercising at 70-75% of one repetition maximum. Accordingly, the depletion of muscle glycogen by resistive exercise training has become an important issue that has to be resolved for professional athletes and active populations. The reason is that a fast recovery will improve the quality of performance at next bout of exercise. Hence, this review will demonstrate resistive training significantly lowers muscle glycogen as endurance training does, and then will consider why muscle glycogen recovery is a key factor of exercise.

Nutritional supplements are a primary strategy to replenish muscle glycogen after exercise. Most important considerations are what nutrients should be consumed and how they work in the human body in order to accelerate and enhance muscle glycogen recovery. Therefore, this review will first give information about major signaling

pathways, which involve the restoration of muscle glycogen. Moreover, information of nutrients that have been shown to greatly effect the anabolic muscle condition will be provided.

GLYCOGEN AS FUEL

Glucose and glycogen are major forms of carbohydrate in the human body and act as primary energy sources during exercise. Their utilizations become an important issue with rising exercise intensity. During low-intensity aerobic exercise, which is around less than 30% of VO_{2max}, carbohydrate oxidation takes 10-15% of total energy expenditure. Furthermore, the largest portion of carbohydrate oxidation comes from plasma glucose with little breakdown of intramuscular glycogen store. However, glycogen utilization dynamically increases to 70-80% of energy production during high-intensity exercise of around 80% of VO_{2max} (Romijn et al. 1993, Hollozy and Kohrt 1996).

As exercise intensity keeps rising, muscle glycogen progressively becomes an important energy fuel, and its stored amount in skeletal muscle seems to be a determinant of athletic performance. Hargreaves and Richter (1988) reported that low muscle glycogen storage might result in a deleterious performance at high and moderate intensity exercise. Therefore, they recommended a carbohydrate-rich diet regimen in the pre-exercise period in order to accumulate intramuscular glycogen storage. The importance of muscle glycogen has been a growing issue in terms of athletic performance because of the evidence that elevated muscle glycogen supports strenuous exercise. Therefore, there has been increased interest in muscle glycogen supercompensation regimen, in which carbohydrate feeding after a glycogen-depleting exercise increases muscle glycogen concentration above the individual basal level (Bergström and Hultman, 1966).

The focus of muscle glycogen research has moved from preparation before training and competition to replenishment after training and competition. Recently, the research of muscle glycogen has investigated the most effective ways of restoring the muscle glycogen within several hours after exercise or between bouts of exercise. The depletion of intramuscular glycogen results in impaired performance at moderate to high intensity exercise (Coyle et al. 1986, Hermansen et al.1965). This problem addresses the necessity of the fast and short-term muscle glycogen recovery in order for the return of performance capacity.

COMPONENTS INVOLVED IN GLYCOGEN REPLENISHMENT

The investigations of the short-term recovery of muscle glycogen have been mainly focused on timing (Ivy et al. 1988a, Levenhagen et al. 2001), the amount of supplementation (Ivy et al. 1988b, Ivy 1998) and type of supplementation to ingest (Burke et al. 1993, Van Loon et al. 2000, Zawadzki et al. 1992). Recent research (Levenhagen et al. 2001) was concentrated on the rate of leg glucose uptake with a carbohydrate-protein supplement, which was provided immediately after or 3 hours after exercise. The rate of leg glucose uptake was roughly 3-fold greater than basal when consumption was immediate, at the immediate consumption, while the 3-hour delayed consumption showed only a 44% increase above basal. Moreover, the studies from Ivy (1998a and 1998) found that immediate ingestion of carbohydrate after exercise promoted muscle glycogen resynthesis more rapidly than delayed ingestion (Ivy et al. 1998a). They also found 1.2 to 1.5g•kg^{-1} body wt of carbohydrate supplemented at 2-hour intervals (Ivy 1998) maximized muscle glycogen storage.

With regard to type of supplement, several studies have found that the combination of carbohydrate with a small amount of protein enhances insulin secretion and glycogen synthesis immediately post-exercise. Although the study of Zawadzki et al. (1992) had a limitation, as the research team did not consider the difference in calories between carbohydrate and carbohydrate-protein supplements, they reported the possibility that ingestion of carbohydrate-protein immediately post-exercise promotes a greater insulin response and muscle glycogen resynthesis than carbohydrate alone. Later research (Ivy et al. 2002, Berardi et al. 2006) suggested a practical approach for short-term muscle glycogen recovery: that a moderate amount of carbohydrate plus a small amount of protein improves muscle glycogen synthesis as much as a high amount of carbohydrate and is effective in reducing the gastric burden caused by large boluses of carbohydrate.

RESISTANCE EXERCISE AND MUSCLE GLYCOGEN UTILIZATION

Research about glycolytic energy metabolism and its resynthesis has predominantly focused on prolonged exercise rather than resistance exercise. This phenomenon is caused by the thought that resistance exercise mainly uses the phosphagen fuels, such as ATP and phosphocreatine, with negligible muscle glycogenolysis. However, recent research has revealed that high-intensity weight bearing exercise also utilizes both the phosphagen fuels and muscle glycogen almost immediately the same as endurance exercise (Goldfarb et al. 1989, Tesch et al.1986). Although evaluation of muscle energy metabolism on weight lifting is limited, several studies have reported that muscle glycogen is a significant energy source following high-intensity resistive exercise and it should be restored after exercise. Muscle glycogen

restoration between bouts may be an important strategy for athletes in order to improve their muscular performance.

Robergs et al. (1991) compared the glycogen depletion following two different intensities of resistance exercise, 75% one repetition maximum (1RM) and 35% 1RM, and the rate of glycogen repletion without supplement after 2 hours post-exercise. In this study, total muscle force development was the same between 75% 1RM with low repetition and 35% 1RM with high repetition. High- and low- intensity resistance exercise showed similar amounts of muscle glycogenolysis to endurance exercise. This finding proved that muscle glycogen utilization of intensive resistance exercise also depends on exercise intensity. Interestingly, the rate of muscle glycogenesis without any supplement was 11.1±3.4 and 7.2±1.3 mmol•kg wet wt^{-1}•h^{-1} in 70% 1RM and 30% 1RM, respectively. This is a greater rate of muscle glycogen synthesis than has typically been shown following endurance exercise when supplementation is provided (Ivy et al. 1988). Although there was no difference in the rate of muscle glycogen recovery with or without supplementation, it is widely known that glucose uptake and muscle glycogen resynthesis is promoted by the availability of glucose and insulin (Ahlborg and Felig, 1976, Maehlum et al. 1978). Therefore, supplying with carbohydrate or carbohydrate plus additional protein after exercise increases the level of glucose and insulin and strongly stimulates muscle glycogen repletion.

Other studies with various post-exercise supplementations have reported faster rates of muscle glycogen recovery. The research from Pascoe et al. (1993) used carbohydrate (1.5g•kg^{-1}) and the same volume of water as a placebo after repetitive 70% 1RM knee extension. This study demonstrated that repetitive high-intensity resistance exercise utilized muscle glycogen as a major fuel source, which was depleted around 30% from the basal level. Post-exercise feeding with carbohydrate enhanced the rate of

muscle glycogen restoration better than feeding with water. After 6 hours of recovery, the amount of recovered muscle glycogen in the carbohydrate ingestion trial was significant and reached 91% of pre-exercise level.

Another study conducted high-intensity resistance exercise with trained subjects and compared carbohydrate alone and isocaloric supplements containing carbohydrate, protein and fat. The study observed plasma insulin and glucose and the rate of muscle glycogen resynthesis (Roy et al. 1998). They found similar patterns of plasma insulin and glucose in both supplements. In addition, there was no difference in the rate of muscle glycogen repletion by both supplements and the rate was similar to the results of Zawadzki et al. (1992), which supplied carbohydrate combined with protein supplement after endurance exercise. These findings also demonstrate that resistance exercise significantly utilizes muscle glycogen as an energy source and the total amount of carbohydrate and energy in a supplement is a critical determinant of the muscle glycogen repletion. Their carbohydrate alone supplement also showed much greater the rate of muscle glycogen resynthesis than that of the previous study (Pascoe et al. 1993). This difference may be due to the training status of the subjects. Roy et al. (1988) recruited highly trained subjects, and it has been demonstrated that highly trained people such as athletes have higher insulin sensitivity and glucose transporter-4 (GLUT4) content compared with those who are untrained (Ebiling et al. 1993). Thus, enhanced insulin sensitivity and GLUT4 content may assist increasing the rate of muscle glycogen recovery.

MECHANISMS OF GLYCOGEN SYNTHESIS AFTER EXERCISE

In the post-exercise period, a sufficient level of substrate, glucose, must be supplied for muscle glycogen recovery. Glucose uptake after exercise is an important first process to restore muscle glycogen. Insulin and substrate availability influence this process. GLUT4 is extensively located in skeletal muscle and insulin normally controls its function. When insulin binds to its receptor, GLUT4 is translocated from an intracellular space to the cell membrane for glucose uptake. Moreover, exercise is able to stimulate the translocation of GLUT4 and is referred to as the insulin-independent pathway (Thorell et al. 1999). It has been suggested that both stimuli, such as insulin and muscle contraction, have additional benefits for the translocation of GLUT4 in the muscle (Lund et al. 1995). The translocation of GLUT4, in response to insulin and muscle contraction may be a crucial determinant of the maximal rate of muscle glycogen replenishment because it supplies sufficient sources of glucose for building muscle glycogen.

Rebuilding muscle glycogen, particularly after exercise, consists of two phases: the rapid and slow phases. The rapid phase is also called the insulin-independent phase because this phase can initiate glycogen storage without insulin. Generally, the rapid phase only persists between 30-60 minutes. This phase solely occurrs when the level of muscle glycogen falls below a certain level, which is 128-150 mmol/kg dw (Price et al. 1994). Glycogen synthase (GS) is considered a rate-limiting factor in glycogen synthesis. Although GS is primarily stimulated by insulin, it has been demonstrated that there is an insulin-independent way to increase GS activity, which explains a tight relationship between the concentration of muscle glycogen and the activity of GS (Nielsen and Wojtaszewski, 2004). For instance, if the concentration of muscle glycogen

lowers after exercise, then GS will become an active form and initiates re-building of muscle glycogen in the skeletal muscle.

The slow phase is also called the insulin-dependent phase. It has been shown that muscle glycogen synthesis in the slow phase is related to increased sensitivity of insulin and muscle glucose uptake. Exercise enhances insulin sensitivity and its status can last over 48 hours depending on carbohydrate supplement and the degree of muscle glycogen (Cartee et al. 1989). Increased insulin sensitivity promotes GLUT4 activity and results in increased muscle glucose uptake. Moreover, insulin-stimulated muscle glucose uptake with a low abundance of muscle glycogen is controlled by activation of protein kinase B (PKB), which is also known as Akt.

Insulin, which is a typical anabolic hormone in the body, strongly mediates muscle glycogen synthesis in part due to its stimulating effect on muscle glucose uptake. In addition, insulin regulates enzymes controlling muscle glycogen synthesis through phosphorylation. After insulin binds to its receptor, a signal that activates phosphatidylinositol 3-kinase (PI3K) occurs and then this signal subsequently phosphorylates Akt/PKB. Phosphorylated Akt/PKB stimulates GLUT4 translocation in response to phosphorylation of AS160, which is recognized as an important signal protein involved in control of GLUT4 translocation (Frosig et al. 2007). Additionally, Akt/PKB regulates GS activity in response to inhibition of glycogen synthase kinase-3 (GSK3) (Cross et al. 1995).

Akt substrate of 160kDa, known as AS160, has been proposed as a significant signaling protein for regulation of GLUT4 traffic to the plasma membrane. This translocation is induced not only by insulin but also by muscle contraction (Sakamoto and Holman, 2008). For instance, the study from Kramer et al. (2006) using mouse tibialis anterior skeletal muscle found that overexpression of AS160-4p reduced the rate

of insulin-dependent glucose uptake (~30%) and of contraction-dependent glucose uptake (~40%) as well. This study suggested that AS160 was a significant factor in controlling glucose uptake. Furthermore, GSK3 plays a crucial role in regulation of GS in skeletal muscle (MacAulay et al. 2005). To stimulate glycogen synthesis requires dephosphorylation of GS. The activation of GS is mainly controlled by the inhibition of GSK3 activity induced by insulin signaling (Suzuki et al. 2001). When GSK3 is phosphorylated by insulin, this inactivates the enzyme and allows activation of GS by dephosphorylation.

The mammalian target of rapamycin (mTOR) is another target protein of Akt/PKB. This is a key protein kinase that regulates protein synthesis by nutrients, such as amino acids, in skeletal muscle. Phosphorylated mTOR activates its downstream proteins including eukaryotic initiation factor 4E binding protein-1 (4E-BP1) and ribosomal protein S6 kinase (p70S6K) (Dennis et al. 1999). It has been reported that p70S6K is powerfully involved in the inactivation of GSK3, which can lead to an increase in muscle glycogen synthesis (Sutherland et al. 1993).

POST-EXERCISE NUTRITIONAL SUPPLEMENTS

Exercise can result in the depletion of energy source such as glycogen within a few hours. The rate of muscle glycogen restoration mainly determines the time of recovery for next-bout of exercise or training. The amount of available substrate in the body after exercise plays a pivotal role in determining the rate of muscle glycogen recovery. Especially, professional athletes or active individuals who perform high-intensity training with short breaks between exercise bouts need a quick recovery.

Therefore, the consumption of nutritional supplements, typically containing carbohydrate and protein, is a predominant strategy for fast recovery in the active population.

Carbohydrate (CHO) supplementation is a major provider of glucose and stimulation of insulin secretion, which are determinants of the rate of muscle glucose uptake. In addition, they assist in building muscle glycogen. Therefore, CHO is highly recommended as a powerful supplement after exercise. Several studies have investigated adequate amounts of CHO for optimizing the rate of muscle glycogen synthesis. The initial study from Ivy et al. (1988) did not observe differences in the rate of glycogen restoration between two doses of CHO consumption, 0.75 or 1.5 g carbohydrate•kg^{-1}•h^{-1}, supplied at 2-h intervals. A subsequent study by Ivy (1998) suggested consuming 1.2 to 1.5 g•kg^{-1} of CHO at 2-h intervals for the purpose of maximally increasing glycogen synthesis. Additionally, this study proposed that more frequent provision of supplements could improve the optimal rate of glycogen storage (Ivy et al. 1998). Later, van Loon et al. (2000) supported this idea that more frequent supplements could accelerate the resynthesis of muscle glycogen at 30-min intervals.

As mentioned above, adequate glucose and insulin availability post-exercise are prime regulators to increase the activation of glucose transport (Kuo et al. 1999) and glycogen synthase (Bak et al. 1991). Moreover, recent studies have reported that ingestion of amino acids with carbohydrates could be beneficial in maximizing the anabolic effect of exercise. The mechanisms of additive anabolic function by amino acids ingestion still remains to be found. However, several researchers have argued that the change in insulin responses is a potential mechanism. Amplified insulin secretion could have a great anabolic effect on the body (Fryburg et al. 1995, Hillier et al. 1998). Therefore, combined supplement with carbohydrate and amino acids has been recommended for short-term and fast muscle glycogen replenishment (Hara et al. 2011,

Ivy et al. 2002. van Loon et al. 2000, Zawadzki et al. 1992). For example, Zawadzki et al. (1992) compared the rate of muscle glycogen synthesis for a 4-hr interval between two supplements, which were carbohydrate (CHO) and carbohydrate plus protein (C+P). This study demonstrated that C+P enhanced the level of plasma insulin leading to a great rate of glycogen synthesis. However, the increased insulin response may have been caused by the higher calorie content of the C plus P supplement compared with the CHO supplement. Further studies that employed isoenergetic CHO and C plus P supplements, such as Ivy et al. (2002) and Hara et al. (2011), have shown that the addition of protein to a carbohydrate supplement promotes muscle glycogen synthesis, although this was not occurred with a greater insulin response. These studies suggest that insulin may not be a major factor increasing the rate of glycogen synthesis with additional protein. Additional protein may play a crucial role in the activation of enzymes regulating glycogen synthesis, including Akt/PKB and/or GS.

SYNERGETIC EFFECTS OF LEUCINE ON GLYCOGEN SYNTHESIS

Branched-chain amino acids (BCAA), especially leucine, are generally known as strong insulinotropic amino acids, and they are frequently added into post-exercise supplements with CHO in order to maximize an insulin secretion. Leucine has been demonstrated to stimulate insulin sensitivity (Zanchi et al. 2012) and anabolic effects through insulin-dependent and/or –independent pathways (Anthony et al. 2000). Enhancing insulin secretion and sensitivity, resulting from leucine and/or additional amino acids intake, is likely to accelerate post-exercise muscle glycogen restoration. Leucine directly activates mTOR, which is a major insulin-independent pathway. In addition, activated mTOR phosphorylates its down stream protein, p70S6K, which is

involved in the inhibition of GSK3, leading to the activation of GS by dephosphorylation (Sutherland et al. 1993).

The effects of leucine on insulin-stimulated glucose uptake have been doubted for a long time (Nichitani et al. 2002, Doi et al. 2005). However, Iwanwaka et al. (2010) provided evidence that leucine could improve contraction-induced, but not insulin-stimulated, glucose uptake (Iwanaka et al. 2010). They suggest possible mechanisms that enhance contraction-induced and attenuate insulin-induced glucose transport. According to the results, leucine directly activated the mTOR/p70S6K pathway in both glucose transport pathways. However, leucine promoted contraction-induced glucose transport via activation of 5'-AMP-activated protein kinase (AMPK), which is a sensor of energy status and is involved in insulin-independent glucose transport. This activation of AMPK was abolished by rapamycin, which is a specific inhibitor of mTOR/p70S6K. Compared with contraction-stimulated glucose transport, insulin-stimulated transport was attenuated by leucine administration via the inhibitory phosphorylation of insulin receptor substrate 1 (IRS 1). It was also diminished by rapamycin. Although these results suggest the opposite effects of leucine on glucose transport, the mechanisms have not yet been identified. Thus, the increase in the rate of muscle glycogen synthesis at post-exercise by administration of leucine could be positively expected with improved anabolic conditions via their insulinotropic character and ability to phosphorylate mTOR/p70S6K.

Β-HYDROXYL-Β-METYLBUTYRATE (HMB) AS A SUPPLEMENT

β-hydroxyl-β-metylbutyrate (HMB) is a metabolite derived from leucine. Leucine is metabolized into α-ketoisocaproate (KIC) and then KIC has two ways to convert into HMB: 1) it directly converts into HMB in the cytosol by α-ketoisocaproate dioxygenase and, 2) it becomes isovaleryl-CoA in the mitochondria by α-ketoacid dehydrogenase before being HMB. Under normal conditions, KIC takes the later pathway to become HMB, therefore only 5% of leucine is converted into HMB (Zanchi et al. 2011). 600g of high quality protein contain approximately 60g of leucine. Thus, this enormous amount has to be consumed in order to make 3g of HMB, which is the recommended daily amount (Wilson et al. 2008). Since this assumption is impractical, intake of HMB as a supplement has become popular. Moreover, any adverse effects, such as liver or renal failure, have not been reported with 3 – 6g/day of HMB.

Anti-catabolic property is the most well known function of HMB. Thus, the studies of HMB supplements are largely divided into two parts; one is related to sports performance, such as resistance or endurance training, another is about therapeutic aspects of HMB which includes clinical conditions, such as cancer, AIDS, and chronic renal failure. The common character of both parts is that skeletal muscle receives extreme stress that can cause proteolysis and muscle wasting. According to the study of Nissen et al. (1996), intake of 3.0g/day of HMB with resistance training for 3 weeks blunted the increase of muscle damage markers, creatine phosphokinase (CPK) and lactate dehydrogenase (LDH). The other studies of HMB (Gallagher et al. 2000a, Panton et al. 2000) also reported a reduction in the indicator of muscle damage and an increase in muscle mass with 3.0g/day of HMB. Several studies have proposed therapeutic effects of HMB. Consumption of HMB with leucine to cancer patients who suffer cachexia improved muscle wasting syndromes (Smith et al. 2005). This positive result may be due

to combined functions with HMB and leucine. HMB could diminish muscle protein degradation while leucine could enhance protein synthesis. Furthermore, HMB lowered tumor growth in tumor-bearing animal studies (Caperuto et al. 2007, Nunes et al. 2008). Although these prevalent properties of HMB have been reported, there is still a lack of studies on aspects of HMB and muscle glycogen recovery.

Most HMB studies have concentrated on the regulation of muscle protein balance. However, the mechanisms on the protein balance still remains to be determined. Based on the studies that revealed possible pathways to increase muscle protein condition, HMB may enhance muscle glycogen restoration following two possible mechanisms: 1) Insulin-like growth factor 1 (IGF-1) gene expression in skeletal muscle and 2) mTOR signaling pathway. Kormasio et al. (2009) reported that cultured cells incubated with HMB expressed IGF-1 nearly twofold more than control cells. IGF-1 is known as a potent activator of Akt/PKB through phosphorylation of PI3K pathway. An activated Akt/PKB leads to the initiation of mTOR pathways, including 4E-BP1 and p70S6K (Bodine et al. 2001). Furthermore, Eley et al. (2007) observed that incubated skeletal muscle cells with HMB significantly enhanced protein synthesis via the activation of mTOR pathways and its down stream proteins. Although HMB studies have only focused on muscle protein synthesis and degradation, HMB may assist in recover of muscle glycogen after high-intensity exercise due to its ability to affect Akt/PKB phosphorylation.

SUMMARY

The rate of muscle glycogen recovery after exercise determines the performance quality of the next bout of exercise or training. Insulin and substrate availability are

significant factors that influence glucose uptake for muscle glycogen restoration. Carbohydrate is the first consideration as a fuel source for a post-exercise supplement, because it provides glucose and increases insulin secretion. Additional protein sources, such as whey protein and leucine, with carbohydrate have been shown to have a positive effect on muscle glycogen restoration. These effects may be caused by the insulinotropic property of protein. In addition, protein could regulate enzymes controlling muscle glycogen synthesis such as Akt/PKB, AS160, GS, and GSK3, but the mechanisms have not been elucidated.

A metabolite of leucine, HMB, has not been seriously considered as a supplement for muscle glycogen replenishment. However, it has anti-catabolic function under severe stressful conditions, including high-intensity exercise and cancer. Furthermore, potential actions on cellular signaling pathways induced by HMB supplementation suggest it could promote muscle glycogen restoration.

APPENDIX A: Glucose Assay

1. Prepare a color reagent (Raichem, 80038, 100mg) and dissolve it with 100ml ddH_2O
2. Set up 12x75mm test tubes and label them as blank, standards, and plasma sample in duplicate
3. Pipette 1.5ml reagent into the each test tube
4. Pipette 20µl of each standard (50mg/dl, 100mg/dl, 150mg/dl, 200mg/d) into tubes in order to make standard curve
5. Add 20µl of plasma samples into corresponding tubes
6. Cover the test tubes with parafilm
7. Incubate the tubes in water bath for 10min at 37°C
8. Turn on the spectrophotometer and set up the wavelength at 500nm
9. After 10min incubation, measure the samples at 500nm

APPENDIX B: Rat Insulin Assay

Day one:

1. Dilute all plasma samples at 1:1 dilution using assay buffer
2. Add 200µl of assay buffer to the non-specific binding (NSB) tubes and 100µl to the reference tubes
3. Add 100µl of standards, samples, or controls to 12x75mm glass assay tubes in duplicate and then add 100µl of hydrated ^{125}I-Insulin to the tubes
4. Add 100µl of rat insulin antibody to all tubes except blank tubes
5. Vortex them and incubate overnight at 4°C after covering the tubes with parafilm

Day two:

1. Add 1ml of cold precipitating reagent to all tubes except blank tubes and NSC tubes
2. Vortex and incubate 20min at 4°C
3. Centrifuge the tubes except blank tubes and drain all tubes for 60sec
4. Count each tube for 2min using gamma counter and determine the concentration based on the standard curve

APPENDIX C: Muscle Glycogen Assay

Prepare stock solutions:

1N KOH (200ml)

Chemical	Amount (g)	FW.
KOH	11.22	56.11

1) Add 11.22g of KOH into 180ml ddH_2O and bring to 200ml with ddH_2O

1N NaOH

Chemical	Amount (g)	FW.
NaOH	8	40

1) Add 8g of NaOH into 180ml ddH_2O and bring to 200ml with ddH_2O

0.3M Sodium Acetate, pH4.8 (500ml)

Chemical	Amount (g)	FW.
Sodium acetate	12.3	82.03

1) Add 12.3g of sodium acetate into 450ml ddH_2O, stir well, and bring to 500ml with ddH_2O

2) Adjust pH to 4.8 with HCl

50% Glacial Acetic Acid (200ml)

Chemical	Amount (ml)
Glacial Acetic Acid	100

1) Add 100ml of glacial acetic acid into 100ml ddH_2O and mix well

Muscle glycogen assay procedure:

Day one

1. Turn on water bath and set to 65-70°C
2. Cut frozen muscle samples around 50-100mg and put into a glass tube containing 1ml 1N KOH
3. Cover the tubes with marbles and incubate at 65-70°C, while shaking, for 20min

4. During 20min incubation, prepare the amyloglucosidase mixture with 0.3M sodium acetate, pH4.8 (0.3M sodium acetate contains 10mg/ml of enzyme) and the enzyme must be put on ice
5. After 20min incubation, vortex the tubes vigorously and incubate for an additional 10min
6. Transfer 100µl of homogenate to glass tubes with 250µl of 0.3M sodium acetate, pH4.8 and then vortex
7. Add 10µl of 50% glacial acetic acid to the tubes
8. Add 250µl of 0.3M sodium acetate, pH4.8, which contained 10mg/ml amyloglucosidase and then vortex well
9. Seal the tubes with parafilm and incubate overnight at RT

Day two

1. Turn on water bath to set at 37°C
2. Prepare Raichem glucose color reagent according to manufacture's instruction
3. Make standards (18.02mg/dl, 9.01mg/dl)
4. Add 25µl of 1N NaOH to the incubated test tubes for neutralization
5. Follow the glucose assay procedure provided by the manufacture
6. Read absorbance of samples at 500nm wavelength using the spectrophotometer

APPENDIX D: Lowery Protein Assay

1. Prepare samples on ice and standards using 5mg/ml bovine serum albumin (BSA) stock following below:

	Water (ml)	Protein (ml)	Protein cont. (mg/ml)
Blank	1.0	0	0
A	1.8	0.2 of stock	0.5
B	0.2	0.8 of A	0.4
C	0.5	0.5 of B	0.2
D	0.5	0.5 of C	0.1
E	0.5	0.5 of D	0.05
F	0.5	0.5 of E	0.025

1) Label duplicate 12x75mm glass assay tubes of each standard and sample

1. 2. Dilute muscle samples with ddH_2O in 1:30 ratio.
2. Make solution A

1) Add 48ml of 2% sodium carbonate (20g/L of 0. 1 NaOH) to a 125ml flask

2) Add 1ml of 1% cupric sulfate and 1ml of 2% sodium potassium tartate to the flask

3) Mix them well

3. Add 0.1ml of standards or samples to the glass tubes
4. Add 1.0ml of Solution A to each tube and incubate for 10min at RT after vortex the tubes
5. Dilute phenol reagent with ddH_2O in 1:2 ratio
6. After 10min incubation, add 0.1ml of the reagent into each glass tube while vortexing the glass tube and then incubate for 30min at RT
7. Turn on the spectrophotometer and let the bulb warm up at wavelength of 750nm.
8. Measure the standards and samples

APPENDIX E: Muscle Homogenization

Muscle Homogenization buffer (100ml, pH7.4)

Reagent	Concentration (mM)	Amount (g)
HEPES	20	0.477
EGTA	2	0.076
NaF	50	0.209
KCl	100	0.746
EDTA	0.2	0.0074
β-Glycerophosphate	50	1.08
DTT	1	0.015
PMSF	0.1	0.00174
Benzamidine	1	0.0157
Sodium vanadate	0.5	0.0092

1. Add all reagents to 80ml ddH_2O, and then adjust pH to 7.4.
2. Bring the volume to 100ml ddH_2O and stir it on a magnetic plate.
3. Store the homogenization buffer at -20°C

Muscle Homogenization procedure:

1. Weight around 80-100mg of muscle samples
2. Dilute the muscle samples to 1:8 in the homogenization buffer.
3. Homogenize the muscle with a glass tissue grinder pestle at 3000rpm by an electrically powered stirrer.
4. Centrifuge muscle samples at 13,000g for 10 min at 4°C
5. Aliquot and store the supematants at -80°C for further analyze

APPENDIX F: Western Blot

Solution preparation:

0.25% Bromophenol Blue Dye

Chemical	Amount (g)
Bromophenol Bule	0.0125

1. Add 0.0125g Bromophenol Blue into 5ml ddH_2O
2. Vortex until dissolved and store at room temperature (RT)

2X Laemmli Sample Buffer (50ml)

Chemical	Amount (ml)
1.25M Tris-HCl (pH6.8)	5
Glycerol	10
20% SDS	5
β-Mercaptoethanol	2.25
0.25% Bromophenol Blue	1.6

1. Add all chemicals into 40ml ddH_2O
2. Stir well until dissolved and bring the volume to 50ml with ddH_2O
3. Store it at RT

30% Acrylamide and 1% Bisacrylamide Mixture (200ml)

Chemical	Amount
Acrylamide	58g
Bisacrylamide	2g

1. Add all chemicals to 150ml ddH_2O
2. Stir well until dissolved on a magnetic plate and bring to 200ml with ddH_2O
3. Filter the solution with Whatman #1 filter paper and store in a dark bottle at 4°C

10% Ammonium persulfate (2ml)

Chemical	Amount (g)
Ammonium persulfate	0.2

1. Add this chemical to 2ml ddH_2O and vortex until dissolved (prepare daily fresh)

1.5M Tris Buffer, pH8.8 (500ml)

Chemical	Amount (g)
Trisbase	90.82

1. Add 90.82g of Trisbase into 400ml ddH_2O and stir it on a magnetic plate
2. Bring to 500ml volume with ddH_2O
3. Adjust pH to 8.8 and store at 4°C

1.0M Tris Buffer, pH6.8 (500ml)

Chemical	Amount (g)
Trisbase	60.57

1. Add 60.57g of Trisbase into 400ml ddH_2O and stir it on a magnetic plate
2. Bring to 500ml volume with ddH_2O
3. Adjust pH to 8.8 and store at 4°C

10% Filtered Sodium Dodecyl Sulfate (SDS) (100ml)

Chemical	Amount (g)
Sodium dodecyl sulfate	10

1. Add the chemical to 80ml ddH_2O and stir it on a magnetic plate
2. Bring to 100ml volume with ddH_2O
3. Filter the solution with Whatman #1 filter paper and store it at RT

20% Filtered Sodium Dodecyl Sulfate (SDS) (100ml)

Chemical	Amount (g)
Sodium dodecycl sulfate	20

1. Add the chemical to 80ml ddH_2O and stir it on a magnetic plate
2. Bring to 100ml volume with ddH_2O
3. Filter the solution with Whatman #1 filter paper and store it at RT

10X Western Blot Running Buffer (2L)

Chemical	Amount (g)
Trisbase	60.56
Glycine	288.4
Sodium dodecycl sulfate	20

1. Add all reagents listed above into 1.5L ddH_2O
2. Stir well until dissolved on a magnetic plate
3. Bring the volume to 2L with ddH_2O and store at RT

10X Tris-Tween Buffered Saline (TTBS), pH7.4 (2L)

Chemical	Amount (g)
Trisbase	24.2
NaCl	175.36
Tween 20	12ml

1. Add all chemicals listed above into 1.5L ddH_2O
2. Stir well until dissolved on a magnetic plate and bring to 2L with ddH_2O
3. Adjust pH to 7.4

0.1% Reversible Ponceau S. Dye (50ml)

Chemical	Amount (g)
Dye-Ponceau S	0.05
Acetic Acid	2.5ml

1. Add 0.05g of Dye-Ponceau S and 2.5ml of Acetic acid into 45ml ddH_2O
2. Vortex well until dissolved and bring to 50ml with ddH_2O
3. Store it at RT

Western Blot Transfer Buffer (2L)

Chemical	Amount (g)
Glycine	28.8
Trisbase	6.04
Methanol	300ml (15%)
Sodium dodecycl sulfate	0.75

1. Add all chemicals into 1.4L ddH_2O and stir well until dissolved on a magnetic plate

2. Bring to 2L with ddH_2O and store it at RT

Western Blot Procedure:

1. Make resolving gel solution (10% resolving gel for 2 gels)

Chemical	Amount (ml)
ddH_2O	4
Acrylamide mix	3.3
1.5M Tris buffer, pH8.8	2.5
20% SDS	0.1
10% APS	0.1
TEMED	0.004

1) Add all chemicals listed above into 50ml test tube.

2) Mix them and fill 3/4 caster with the resolving gel solution (around 4.5ml/gel)

3) Put 200µl butanol on the resolving gel and allow 30 min for the gel to polymerize

4) After gel polymerized, pour butanol and smoothly wash the caster with ddH_2O

2. Make stacking gel solution (for 2 gels)

Chemical	Amount (ml)
ddH_2O	3.4
Acrylamide mix	0.83
1.0M Tris buffer, pH8.8	0.63
20% SDS	0.05
10% APS	0.05
TEMED	0.005

1) Add all chemicals listed above into 15ml test tube and well mixed

2) Fill the caster with stacking gel solution then put combs into place

3) Allow 30min for stacking gel to polymerize

3. Prepare samples

1) Take the samples out of -80°C and thaw them on ice

2) Dilute the samples to 1:2 with sample buffer in the microfuge tubes

3) Vortex and place in 90-95°C water for 10min

4. Prepare 1X Running buffer

1) Add 100ml 10X Running buffer into 900ml ddH_2O and stir on a magnetic plate

5. Remove the combs and assemble gel apparatus and then fill inner and out chamber with 1X Running buffer
6. Load 10µl of standard, 12-20µl of the samples, or 10µl of molecular weight marker to corresponded gel lane
7. Electrophoresis at 130V for 1h 30min
8. After the electrophoresis, prepare for wet-transfer buffer described as above
9. Cut 9*6cm rectangle Nitric Cellular Membranes and label them
10. Carefully separate casing plates, remove the stacking gel, and place the resolving gel in the wet-transfer buffer for 5min

11. Assemble the transfer sandwich cassette in the transfer buffer according to the orders: black plate➔spongy pad➔filter paper➔the resolving gel➔the membrane➔filter paper➔spongy pad➔clear plate
12. Electrophoresis at 90V for 1h 30min
13. During the transfer, prepare 1X TTBS

1) Add 100ml 10X TTBS into 900ml ddH_2O and stir on a magnetic plate

14. Prepare 7% non-fat dry milk (NFDM) (30ml/membrane)

1) Add 4.2g NFDM into a 100ml beaker containing 60ml 1X TTBS and stir it on a magnetic plate

15. After transfer, dissemble the sandwich, stain each membrane with Ponceau S. for 5 sec, and then put membrane into 1X TTBS on a shaker to wash out Ponceau S. on the membrane
16. Put the washed membrane into a container with 7% NFDM for 20min with gently shaking
17. During blocking, prepare primary antibody with 2% NFDM
18. After blocking, wash three times for 5min with 1X TTBS
19. Incubate the membrane with the primary antibody overnight at 4°C with gently shaking
20. After overnight incubation, wash 3x5min with 1X TTBS
21. Incubate the membrane with secondary antibody for 1h at RT with gently shaking
22. After secondary incubation, wash 3x5min with 1X TTBS
23. Visualize protein bands using an ECL detection kit and Bio-Rad ChemiDoc detection system following the manufacturer's instructions
24. Quantify the density of the bands using Quantity One Analysis software

APPENDIX G: Modified Krebs-Henseleit Bicarbonate (KHB) Buffer

Modified Krebs-Henseleit Bicarbonate (KHB) Buffer-stock solutions

Stock 1: 10X (500ml)

Chemical	Final Cont.	Amount (g)	FW.
NaCl	1.16M	33.895	58.44
KCl	46mM	1.7145	74.56
KH_2PO_4	11.6mM	0.789	136.1
$NaHCO_3$	253mM	10.625	84.01

1. Add all chemicals listed above into 400ml ddH_2O and stir well until dissolved on a magnetic plate
2. Bring to 500ml with ddH_2O and store it at 4°C

Stock 2: 10X (500ml)

Chemical	Final Cont.	Amount (g)	FW.
$CaCl_2 \cdot 2H_2O$	25mM	1.8375	147
$MgSO_4 \cdot 7H_2O$	11.6mM	1.4295	246.48

1. Add all chemicals listed above into 400ml ddH_2O and stir well until dissolved on a magnetic plate
2. Bring to 500ml with ddH_2O and store it at 4°C

Make 500ml of KHB buffer:

1. Mix 50ml of Stock 1 with 380ml ddH_2O
2. Add 50ml of Stock 2 into Stock 1 solution and adjust pH to 7.4
3. Bring to 500ml with ddH_2O

APPENDIX H: Raw Data

Plasma Glucose

# of rats	Treatment	0min (mM)	45min (mM)	AUC (mMx45min)
5	CON	7.43		
16	CON	3.92		
22	CON	7.55		
26	CON	6.64		
28	CON	9.90		
34	CON	8.31		
39	CON	8.89		
2	CHO	6.95	7.39	9.81
3	CHO	6.49	7.60	25.00
13	CHO	6.80	7.87	24.11
15	CHO	8.56	8.14	-9.51
29	CHO	7.66	8.29	14.34
33	CHO	7.99	8.21	4.95
35	CHO	7.77	9.56	40.25
4	CP	8.87	9.00	2.93
14	CP	7.92	8.37	10.13
21	CP	7.57	7.63	1.46
24	CP	7.99	7.43	-12.44
31	CP	7.97	7.67	-6.74
32	CP	7.00	8.15	25.89
38	CP	7.75	7.20	-12.28
6	CPH	5.55	8.11	57.61
17	CPH	9.39	8.26	-25.35
19	CPH	7.95	8.17	4.95
20	CPH	6.55	8.33	40.09
23	CPH	8.12	8.39	5.87
30	CPH	7.04	9.18	48.35
36	CPH	7.38	9.39	45.18
1	CPHL	8.81	7.67	-25.85
10	CPHL	7.55	6.76	-17.66
18	CPHL	7.56	7.41	-3.29
25	CPHL	7.03	6.27	-17.05
27	CPHL	8.72	7.54	-26.69
37	CPHL	7.65	7.72	1.46
42	CPHL	7.68	7.03	-14.69

Plasma Insuln

# of rats	Treatment	0min (pM)	45min (pM)	AUC (pMx45min)
5	CON	147.38		
16	CON			
22	CON	247.59		
26	CON	257.58		
28	CON	545.80		
34	CON	406.68		
39	CON	381.20		
2	CHO	268.60	318.18	1115.70
3	CHO	95.04	250.69	3502.07
13	CHO	172.18	308.54	3068.18
15	CHO	224.52	272.38	1076.96
29	CHO	77.82	288.91	4749.48
33	CHO	215.56	431.47	4857.95
35	CHO	273.76	468.32	4377.58
4	CP	318.18	726.24	9181.30
14	CP	462.47	515.84	1200.93
21	CP	205.23	300.28	2138.43
24	CP	299.93	298.55	-30.99
31	CP	227.96	384.30	3517.56
32	CP	89.88	391.18	6779.44
38	CP	421.14	714.19	6593.49
6	CPH	197.66	238.64	922.00
17	CPH	283.75	361.57	1751.03
19	CPH	214.19	312.67	2215.91
20	CPH	209.75	263.77	1215.65
23	CPH	528.24	648.07	2696.28
30	CPH	200.07	390.84	4292.36
36	CPH	272.38	737.60	10467.46
1	CPHL	176.65	591.60	9336.26
10	CPHL	370.87	428.03	1286.16
18	CPHL	152.89	660.81	11428.20
25	CPHL	235.19	625.34	8778.41
27	CPHL	367.42	546.14	4021.18
37	CPHL	397.38	524.45	2858.99
42	CPHL	369.83	449.38	1789.77

Muscle glycogen concentration

# of rats	Treatment	cont. (µg/100µl)	mg/g muscle	µmole/g muscle
5	CON	4.80	4.01	22.26
16	CON	2.49	1.86	10.33
22	CON	4.46	3.22	17.87
26	CON	4.50	3.53	19.59
28	CON	4.89	3.57	19.79
34	CON	4.76	3.08	17.11
39	CON	3.70	2.93	16.28
2	CHO	4.40	3.21	17.82
3	CHO	4.57	3.09	17.13
13	CHO	4.42	3.47	19.23
15	CHO	5.65	3.78	20.95
29	CHO	4.23	2.86	15.87
33	CHO	4.82	3.29	18.28
35	CHO	4.15	2.96	16.43
4	CP	4.80	3.63	20.14
14	CP	6.00	4.06	22.51
21	CP	5.30	3.70	20.52
24	CP	4.36	3.25	18.06
31	CP	3.90	3.06	16.97
32	CP	4.09	3.24	18.01
38	CP	4.80	3.63	20.14
6	CPH	3.52	2.35	13.05
17	CPH	4.25	2.87	15.94
19	CPH	4.86	3.77	20.90
20	CPH	3.98	2.84	15.77
23	CPH	6.76	4.67	25.90
30	CPH	4.44	3.14	17.40
36	CPH	4.57	3.58	19.88
1	CPHL	5.28	3.46	19.17
10	CPHL	4.69	3.28	18.18
18	CPHL	4.67	3.19	17.71
25	CPHL	5.49	3.88	21.51
27	CPHL	4.53	3.30	18.33
37	CPHL	5.63	3.80	21.10
42	CPHL	5.36	3.62	20.10

Western Blot

# of rats	Treatment	p-Akt/STD	p-mTOR/STD	p-AS160/STD
5	CON	0.46	0.94	0.42
16	CON	0.15	0.66	0.49
22	CON	0.29	0.77	0.52
26	CON	0.18	1.21	0.38
28	CON	0.35	1.29	0.79
34	CON	0.46	1.09	0.54
39	CON	0.46	1.23	0.39
2	CHO	0.28	0.70	0.28
3	CHO	0.18	0.95	0.32
13	CHO	0.44	0.75	0.59
15	CHO	0.17	0.49	0.21
29	CHO	0.52	1.23	0.38
33	CHO	0.58	1.11	0.24
35	CHO	0.54	1.08	0.57
4	CP	0.36	0.87	0.70
14	CP	0.55	0.61	0.31
21	CP	0.36	0.91	0.61
24	CP	0.24	1.03	0.54
31	CP	0.28	1.14	0.53
32	CP	0.14	0.86	0.59
38	CP	0.56	1.05	1.19
6	CPH	0.23	1.03	1.08
17	CPH	0.22	0.64	0.30
19	CPH	0.41	0.79	0.43
20	CPH	0.23	0.71	0.31
23	CPH	0.23	0.78	0.44
30	CPH	0.45	0.61	0.56
36	CPH	0.51	0.86	0.67
1	CPHL	0.20	1.00	1.05
10	CPHL	0.24	0.73	0.96
18	CPHL	0.36	0.79	0.84
25	CPHL	0.22	0.83	1.17
27	CPHL	0.48	0.86	0.54
37	CPHL	0.49	0.94	0.45
42	CPHL	0.44	0.50	0.67

# of rats	Treatment	p-GSK3α/STD	p-GSK3β/STD	p-GS/STD	α-tubulin/STD
5	CON	0.88	0.69	1.01	4.28
16	CON	0.50	0.49	0.88	7.69
22	CON	0.47	0.43	0.47	3.32
26	CON	0.64	0.58	1.35	2.06
28	CON	0.90	1.52	0.82	3.19
34	CON	1.04	1.62	0.72	2.87
39	CON	0.70	1.03	0.58	2.35
2	CHO	0.68	0.61	0.95	2.22
3	CHO	0.59	0.62	0.67	4.02
13	CHO	0.64	0.50	1.18	5.28
15	CHO	0.53	0.47	0.66	4.22
29	CHO	1.13	1.55	0.88	6.52
33	CHO	1.11	1.55	0.65	1.67
35	CHO	0.95	1.59	0.52	1.96
4	CP	0.63	0.57	0.78	3.96
14	CP	0.55	0.55	1.47	5.87
21	CP	0.53	0.44	1.34	7.51
24	CP	0.57	0.65	1.08	4.69
31	CP	0.87	1.50	0.73	2.70
32	CP	1.03	1.19	0.62	2.02
38	CP	1.12	1.39	0.65	2.34
6	CPH	0.85	0.63	1.19	7.29
17	CPH	0.63	0.71	0.75	4.45
19	CPH	0.59	0.58	1.19	4.03
20	CPH	0.67	0.51	0.81	3.15
23	CPH	0.57	0.49	1.34	5.48
30	CPH	0.76	1.07	0.56	1.88
36	CPH	1.16	1.45	0.50	2.70
1	CPHL	0.80	0.71	1.22	5.41
10	CPHL	0.59	0.52	1.37	7.42
18	CPHL	0.65	0.55	1.09	4.97
25	CPHL	0.60	0.59	1.32	4.95
27	CPHL	1.19	2.08	1.02	3.26
37	CPHL	1.24	1.90	0.80	2.60
42	CPHL	0.74	1.02	0.49	2.16

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